Complement and CD14: Roles in *Escherichia coli*-induced inflammation in the pig

by

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NORD

Se oftere mot nord.
Gå mot vinden, du får rødere kinn.
Finn den ulendte stien. Hold den.
Den er kortere.
Nord er best,
Vinterens flammehimmel, sommernattens solmirakel.
Gå mot vinden. Klyv berg.
Se mot nord.
Oftere.
Det er langt dette landet.
Det meste er nord.

Rolf Jacobsen

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List of papers

This Thesis is based on the following papers:

I **Thorgersen EB**, Ghebremariam YT, Thurman JM, Fung M, Nielsen EW, Holers VM, Kotwal GJ, Mollnes TE. Candidate inhibitors of porcine complement.

Mol Immunol. 2007 Mar; 44(8):1827-34.

II **Thorgersen EB**, Pharo A, Haverson K, Axelsen AK, Gaustad P, Kotwal GJ, Sfyroera G, Mollnes TE. Complement- and CD14-inhibition attenuate *Escherichia coli*-induced inflammatory response in porcine whole blood. Infect Immun. 2009 Feb; 77 (2): 725-732.

III **Thorgersen EB**, Macagno A, Rossetti C, Mollnes TE. Cyanobacterial LPS antagonist (CyP)-a novel and efficient inhibitor of *Escherichia coli* LPS-induced cytokine response in the pig.

Mol Immunol. 2008 Aug; 45(13):3553-7.

IV Castellheim A, **Thorgersen EB**, Hellerud BC, Pharo A, Johansen HT, Brosstad F, Gaustad P, Brun H, Fosse E, Tønnessen TI, Nielsen EW, Mollnes TE. New biomarkers in an acute model of live *Escherichia coli*-induced sepsis in pigs.

Scand J Immunol. 2008 Jul; 68(1):75-84.

V **Thorgersen EB**, Hellerud BC, Nielsen EW, Barratt-Due A, Fure H, Lindstad JK, Pharo A, Fosse E, Tønnessen TI, Johansen HT, Castellheim A, Mollnes TE. CD14-inhibition efficiently attenuates early inflammatory and hemostatic responses in *Escherichia coli*-sepsis in pigs. Submitted.

Abbreviations

ANOVA, Analysis of Variance

AP, Alternative Pathway

ARDS, Acute/Adult Respiratory Distress Syndrome

AU, Arbitrary Units

C1-INH, C1-inhibitor

CD, Cluster of Differentiation

CFU, Colony Forming Units

CLP, Cecal Ligation and Puncture

CP, Classical Pathway

CyP, *Cyanobacterial* Product

CR, Complement Receptor

CRP, C-reactive protein

CVP, Central Venous Pressure

DAMP, Danger-Associated Molecular Pattern

DNA, Deoxyribonucleic Acid

E. coli, *Escherichia coli*

EDTA, Ethylenediaminetetraacetic acid

EIA, Enzyme Immunoassay

ELISA, Enzyme-Linked Immunosorbent Assay

FUT-175, Futhan-175

GPI, Glycosylphosphatidylinositol

HAIGG, Heat-Aggregated Immunoglobulin G

HMGB-1, High Mobility Group Box-1

I/R, Ischemia-Reperfusion

IL, Interleukin

IP, Intrapulmonary

IV, Intravenously

K. pneumoniae, *Klebsiella pneumoniae*

L, Litre

LAL, *Limulus* Amebocyte Lysate

LBP, LPS-Binding Protein

LP, Lectin Pathway

LPS, Lipopolysaccharide

MAC, Membrane Attack Complex

MAP, Mean Arterial Pressure

MAS, Meconium Aspiration Syndrome

MD-2, Myeloid Derived protein-2

mL, Millilitre

MMP, Matrix Metalloproteinase

MODS, Multiple Organ Dysfunction Syndrome

MPAP, Mean Pulmonary Arterial Pressure

Nm, Nanometre

PAI-1, Plasminogen Activator Inhibitor-1

PAMP, Pathogen-Associated Molecular Pattern

PAOP, Pulmonary Arterial Occlusion Pressure

PCR, Polymerase Chain Reaction

PIRO, Predisposition, Insult (Infection), Response, Organ dysfunction

PRR, Pattern-Recognition Receptor

rhAPC, Recombinant Human Activated Protein C

RES, Reticuloendothelial System

S. typhosa, *Salmonella typhosa*

SIRS, Systemic Inflammatory Response Syndrome

SPICE, Smallpox Inhibitor of Complement Enzymes

TAT, Thrombin-Antithrombin complex

TCC, Terminal Complement Complex

TF, Tissue Factor

TLR, Toll-Like Receptor

TNF-α, Tumor Necrosis Factor-α

VCP, Vaccinia Complement Control Protein

VEGF, Vascular Endothelial Growth Factor

WBC, White Blood Cell Count

1 Introduction

1.1 Inflammation

1.1.1 Innate immunity

The innate immune system forms the early barriers in the defense against invading microorganisms. Our knowledge of the cells, cascade systems and structures that forms the innate immune system are still expanding, but traditionally cells like granulocytes, mast cells, macrophages/monocytes, natural killer cells and dendrittic cells, as well as the complement protein cascade system are often mentioned as innate (1, 2). Innate immunity depends upon germline-encoded receptors and does not exhibit features like clone selection and clone expansion which are hallmarks of the adaptive immune system. The system relies on detection of conserved or common features of the invaders. These structures are often refered to as pathogen-associated molecular patterns (PAMPs) and the innate immune recognition proteins are accordingly called pattern-recognition receptors (PRR) (3-6). An important set of PRRs are the Toll-like receptors discussed later. The recognition of these "patterns" makes the system fast acting; the respons is immediate while adaptive immune responses take days. Innate immune receptors do not only detected foreign structures (non-self) but also damaged tissue of the host (damaged self). A proposed name for structures arising from damaged tissues is alarmins (7). According to this, the system recognize "danger" whether it is foreign (PAMPs) or damaged self (alarmins) and the structures recognized could collectively be called danger-associated molecular patterns (DAMPs) (7, 8). The term DAMP, is often refered to as damaged-associated molecular patterns, and was originally postulated as an endogenous counterpart to PAMPs arising from damaged self (9-11). In this Thesis, however, I will use the term DAMP as danger-associated molecular patterns which include both exogenous PAMPs and endogenous damaged self (alarmins). Both PAMPs and damaged self (alarmins) are operative in innate immune recognition and research over the past 10 - 15 years suggest they both can be applied as important in initiating innate immune responses (12, 13).

The “phenotype” of most induced responses resulting from innate immune recognition, whether the recognition is endogenous (damaged self) or exogenous (microorganisms) of origin, is inflammation. The main function of inflammation is to resolve the infection or repair the damage and subsequently return to a state of homeostasis (14). Macrophages/monocytes and granulocytes and other cells secrete a range of inflammatory mediators collectively called cytokines. Cytokines mediate their effect locally in the tissues as well as systemically. They can act in autokrine, paracrine or endocrine manner, inducing their effects on the cells who released the cytokine, on adjacent cells or on distant cells respectively (2). The cytokines induce a range of effects like chemotaxis, upregulation of receptors on cells, trigger blood clotting, and capillary leak (15-17). Effects of the cytokines along with effects of a range of other mediators like lipid-metabolites and reactive-oxygen species “create” the state of inflammation (14).

1.1.2 Toll-like receptors

The german word for “great” is “Toll”. And indeed, the detection of the Toll-like receptors (TLRs) has been a major advance and “great” for understanding of innate immune recognition and activation. The precence of receptors with functions like the TLRs was predicted years before they were discovered (3). In particular, the search for a transmembrane receptor for lipopolysaccharide (LPS) of Gram-negative bacteria was intense. The membrane-bound CD14 was found to bind LPS and mediate LPS-effects (18, 19), but as the molecule was glycosylphosphatidylinositol (GPI)-anchored and not transmembrane it was an enigma how the signalling to the inside of the cell could occur (20). Toll was first discovered in the fruit-fly *Drosophila* as a regulator of dorsal-ventral pattern formation in the fly embryo (21, 22). Later it was found that Toll was responsible for the anti-fungal response in adult *Drosophila*, and thereby was an important part of the immune defence in the fly (23). Soon Toll was also found in humans (24), and after that TLR4 was discovered to be the long-sought transmembrane LPS-receptor (25). The TLR familiy now consist of 13 members in mice and humans together (26). All of the TLRs with known function recognize, as predicted, conserved molecules or patterns like membrane-constituents, DNA or RNA of a variety of microbes (27). Interestingly, recent findings indicate that TLRs not only recognize

exogenous microbial ligands (PAMPs) but various endogenous ligands (alarmins) as well (27, 28). Endogenous ligand recognition is most extensively studied for TLR4, among others heparan sulphate shedded from damaged endothelium (29), heat-shock proteins and fibrinogen are recognized by the receptor (30, 31). All the TLRs' ligands, wether they are exogenous or endogenous, named either PAMPs or alarmins, signals danger to the host (27). The TLRs with theire wide range of exogenous and endogenous ligands are thereby one of the most important PRRs of the innate immune system and equally important in sterile induced danger, like ischemia-reperfusion (I/R) injury (32, 33), as in danger induced by microbes.

1.1.3 Toll-like receptors, lipopolysaccharide and Gram-negative bacteria

LPS is an important constituent of the outer membrane of Gram-negative bacteria, it mediates many of the proinflammatory effects of these pathogens and is thereby an important PAMP. After the detection of the TLRs, the TLR4 was shortly after recognized as the transmembrane LPS receptor in mice as mentioned earlier (25). However, other TLRs, in particular TLR2 was found to bind LPS and induce LPS signalling as well. Indeed, TLR2 was thought to be the human receptor for LPS (34, 35). Further evidence, however, confirmed that the TLR4 was the LPS receptor in mice (36), and in humans TLR2 signalling by LPS was shown to be due to impurities of the LPS preparations used (37, 38). TLR4 is now established as the sole LPS receptor (39). TLR4 by itself is not enough to bind LPS and trigger appropriate downstream intracellular signalling. CD14 was detected earlier than the TLRs and found to bind the complex of LPS bound to the plasma LPS-binding protein (LBP) (18, 40). With the detection of TLR4 as the transmembrane LPS signal conduit, CD14 was recognized as an important and necessary co-receptor with high specificity for LPS (41). Additionally, a soluble protein called myeloid-derived factor-2 (MD-2) which associate with the extracellular domain of TLR4, was detected and shown to be important in LPS induced TLR4 signalling (42). When bound to CD14, LPS is transferred to the TLR4/MD-2 complex (43) and once LPS is bound to TLR4, the Toll-receptors oligomerize and trigger the downstream intracellular cascade (27, 44). At least two intracellular signalling pathways downstream of TLR4 exist, the MyD88-dependent and – independent pathways (45), and the signalling cascade culminates on the nuclear

transcription factor NF-κB (46). CD14 is shown to be required for functional MyD88-independent LPS-mediated signalling (47), and CD14/TLR4/MD-2 and TLR4/MD-2 without CD14 are shown to discriminate and react differently to various types of LPS (48). As TLR4 is widely distributed, while CD14 expression is by far limited to myeloid-derived cells, these LPS discriminating properties suggest that different types of LPS will activate various cells differentially (48). When activated, the cells produce a range of proinflammatory cytokines which drive the inflammatory resonse (49, 50). Interestingly, the key downstream molecule MyD88, is shown to be more homologous between humans and pigs than humans and mice, which may be of importance studying and interpreting LPS-signalling data in these model animals, suggesting the pig to be the better suited (51).

1.1.4 The complement system

The complement system consists of more than 30 proteins mainly found in plasma but also bound to cell surfaces (52). It is named so because it was identified as a heat-labile component in serum "complementing" the heat-stabile antibodies in the killing of bacteria (53). The system is ancient and is both preserved and developed in evolution (54). It is a cascade system in plasma, and is activated by three distinct pathways: The classical pathway (CP), the lectin-pathway (LP) and the alternative pathway (AP) (55). The CP is activated by C1q binding to immunoglobulins (52), particularly IgM, but also other molecules like C-reactive protein (CRP) and phosphatidylserine with importance for clearance of apoptotic cells (56, 57). The LP is activated by mannose-binding lectin and ficolins binding to in particular carbohydrate structures (58, 59). The AP pathway is continuously activated by hydrolysis of C3 and triggered by a range of different compounds like lipids, carbohydrates and proteins (60). In the 50's properdin was proposed as an initiator of this pathway and recent evidence supports these early findings (61, 62). Another and very important function of the AP is amplification of the complement respons initiated by the other two pathways (63). All the recognition molecules of the complement system (e. g. C1q, MBL, ficolins and properdin) are PRRs, as they recognize PAMPs and alarmins and mediate the danger signals through complement-activation (8).

The cascade initiated by all three pathways leads to formation of the C3-convertase and thereby the C5-convertase and the end-product of the final common pathway is the terminal C5b-9 complement complex (TCC). TCC exists both in the fluid-phase or inserted into membranes, where it is often called the membrane-attack complex (MAC). The CP and LP both activate serine proteases which cleaves C4 and C2 and forms the C3 convertase C4b2a. C4b2a cleaves C3 to C3a and C3b and forms the C5 convertase C4b2a3b. The AP forms C3b by the mentioned hydrolysis. C3(H_2O) binds factor B which is then cleaved by factor D to C3(H_2O)Bb. C3(H_2O)Bb cleaves C3 to C3a and C3b. C3b binds factor B and the second C3 convertase, C3bBb forms. The alternative pathway C5 convertase, C3b3bBb is then formed by binding another C3b. C5 is cleaved by either C5 convertase and forms C5a and C5b. C5b binds C6 and the complex bind C7, C8 and nC9 and forms the TCC (52, 64).

The main function of the complement system is to detect and kill microorganisms, as well as maintain homeostasis through clearance of apoptotic cells and cell debris, and tissue regeneration (65-68). Opsonization by C3b leading to phagocytosis is the most important mechanism, but for some pathogens like Neisseria-species, killing by direct lysis by the MAC is equally important (69). The products of the complement system have however a number of other effects. C5a is an important mediator of inflammation as discussed later, and TCC and other complement proteins are involved in cell death (apoptosis and necrosis), clearance of apoptotic cells and cell activation (66, 70). A number of soluble and cell-bound regulatory proteins act to inhibit the complement system, keeping it under tight control (71).

1.1.5 Complement and inflammation

Complement activation initiated by any of the three pathways leads to inflammation (72, 73), with the exception of the role of complement in removal of apoptotic cells (70). The primary inflammatory effects by the complement system could be dived into receptor- and non-receptor mediated effects. The secondary inflammatory effects induced by complement develop during processes like C3b-opsonized phagocytosis.

Important receptor-mediated inflammatory effects come from C3a and C5a, called anaphylatoxins, and are produced as split products of the complement cascade and bind to specific reseptors on cells (74). C4a is also traditionally recognized as one of the anaphylatoxins, but no receptor for the molecule or biological effect is known (74). A recent report, however, has shown direct antimicrobial properties of C4a, which may have importance in the antimicrobial defence (75). C3a has been implicated in inflammation through binding to its receptor C3aR, which is particular abundant expressed on mast cells and eosinophils (65). A particular focus concerning C3a-mediated inflammatory effects, has been on lung diseases like asthma and ARDS (76). C3a is also found to have importance in other inflammatory conditions, like I/R-injury in the kidneys (77). C5a is the key inflammatory mediator of the anaphylatoxins and probably of all the complement components. It is a peptide with a molecular weight of 11 kDa and it binds to the C5aR (CD88) and to the C5L2 receptor (78, 79). It is rapidly degraded to the less potent C5a desArg which also binds to C5aR and C5L2 (79). The effects of binding of C5a or C5a desArg to their receptors depend on the cell type expressing them. C5a is known to have a range of effects on the innate immune cells in particular neutrophils and macrophages: it is a chemoattractant and enhance expression of adhesion molecules on the cells both of which are important to direct the phagocytes from the blood to the site of infection/injury (80, 81); the molecule induce phagocytosis and the consecutive oxidative burst to kill the phagocytized microorganisms (64), and it releases enzymes from intracellular granules as well as induces production and release of cytokines (82-84). The effect of C5aR binding of C5a is most extensively studied while the role of C5L2 is less well understood, although recent studies have indicated both important pro- and anti-inflammatory effects of the C5L2 receptor (85, 86). C5aR is also expressed on endothelial cells, where binding of C5a leads to proinflammatory effects like upregulation of the adhesion molecule P-selectin (87), as well as production of cytokines and the important inductor of coagulation, tissue factor (TF) (88, 89).

The non-receptor mediated primary inflammatory effect of complement activation is mediated by TCC (MAC) inserted into membranes. The insertion leads to Ca^{2+}-influx which acts as a second messenger, and effects like adhesion molecule upregulation and release of inflammatory mediators may be induced (70).One study has proposed a primary receptor-mediated inflammatory effect by the soluble TCC via binding to a

vitronectin receptor (90), but the possible role of this interaction has not been further described.

The complement system is implicated in the pathogenesis of both acute and chronic inflammatory diseases. The role of complement activation has been shown to play an important part in the pathogenesis of acute inflammatory conditions like I/R-injury (91), meconium aspiration syndrome (MAS) (92), trauma (93) and sepsis (94). In addition complement is involved in chronic inflammatory diseases like glomerulonephritis and rheumatoid arthritis (95-97).

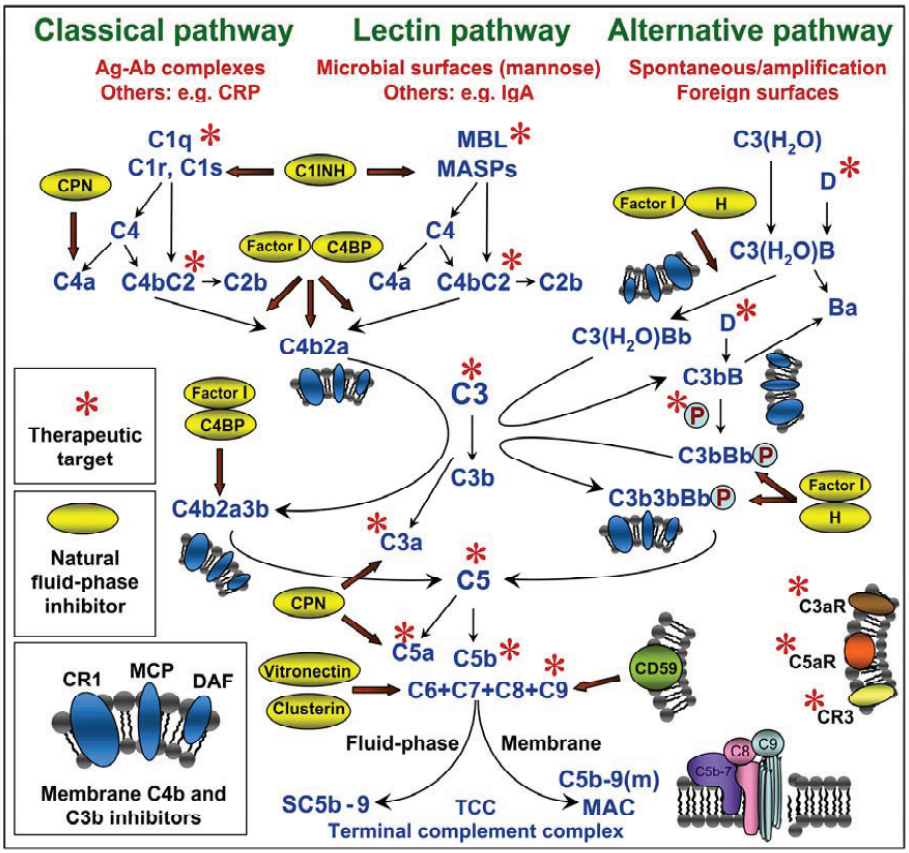

Figure 1. **The Complement System** (Mollnes TE, Song WC, Lambris JD 2002 Complement in inflammatory tissue damage and disease. Trends Immunol 23:61-64, with permission from Elsevier)

1.2 Sepsis

1.2.1 Definition

Sepsis could be defined as systemic inflammatory response syndrome (SIRS) caused by infection (98), as systemic inflammation could be induced by a variety of severe conditions other than infections, like burns and trauma. The initial SIRS definition was two or more of: temperature > 38 ºC or < 36 ºC, heart rate > 90 beats/min, respiratory rate > 20 breaths/min or $PaCO_2$ < 4.3 kPa, WBC > 12 x 10^9/ L or < 4 x 10^9/ L or > 10 % immature forms (98). In this concensus article definitions of categories based on severity in sepsis were also proposed. The categories suggested were sepsis, severe sepsis, septic shock and multiple organ dysfunction syndrome (MODS) (98). The sepsis definition was, however, found to be overly sensitive and non-spesific and a new set of more detailed criteria was therefore proposed, for details see Levy et al. (99). Another staging of sepsis called PIRO (Predisposition, Insult (infection), Response, and Organ dysfunction) was also presented (99). The challenge of a clear definition and staging of sepsis is however still present, a particular demanding area is inclusion of homogenic sepsis populations in clinical trials (100). Further improvement and revision of the present definitions of sepsis seems to be mandatory (101).

1.2.2 Epidemiology – Sepsis and Gram-negative sepsis

Recent studies from European countries show an incidence of sepsis in the adult population of 75 to 350 per 100,000 per year (102, 103). Figures from the USA show an incidence of 130 to 240 per 100,000 per year (104, 105), and 1.3% of all hospitalizations in the US over a 22-year period was due to sepsis (104). The mortality rates range from 10 % for sepsis in general to 50 % for severe sepsis (16, 102, 103).

Gram-negative bacteria as the causative pathogen in sepsis have declined in recent years, but is still a major cause of the syndrome, counting for approximately 25 to 40 % of all cases (104, 106). *Escherichia coli* (*E. coli*) is on of the most frequent causative Gram-negative bacteria , with a frequency of 13 % of all cases in the Sepsis Occurrence in Acutely Ill Patients (SOAP) study (107). A frequency of *E. coli* of up to 27 % of the total causative pathogens in sepsis has been reported (106). *E. coli* strains showing

multidrug resistans to antibiotics are an increasing problem and a major concern in infectious disease and sepsis care (108).

1.2.3 Risk factors

The occurrence of sepsis in adults peaks in the sixth decade of life (102, 103). Factors that can predispose to sepsis are cancer, primary, acquired or pharmacologically induced immunodeficiency, chronic organ failure, and iatrogenic factors like surgery or catheters (106). Pulmonary, gastrointestinal, urinary and skin infections are the leading origins leading to sepsis (103). However, the origin of the infection is often hard to reveal. Recurrently found clinical risk factors independently associated with mortality in sepsis are adequacy of antibiotics, underlying disease, source and type of infection, presence of shock, need for vasopressors, multiple organ failure, and neutropenia (109). Research on genetic factors predisposing to sepsis has revealed polymorphisms that may predispose to or protect against the syndrome, but unfortunately many of these studies have been underpowered and often provided weak indications or contradictory results (110). Despite this, interesting data has emerged from the genetics field. For instance is quantitative or functional deficiency of on of the complement systems PRR, MBL, associated with development of sepsis (111).

The search for biomarkers that can help diagnose sepsis and predict the outcome of the syndrome has been intense. Long lists of candidates have been made, see for instance Lever et al. (112), and new ones are added rapidly to those lists. As sepsis is a syndrome and involves almost all defense systems in the body, the complexity is enormous and finding single mediators to predict the prognosis or outcome have thus been hard.

1.2.4 Treatment

Treatment of sepsis and in particular treatment of severe sepsis and septic shock is multifactorial and may be divided into two main cathegories: antibiotics and supportive therapy. International guidelines founded on consensus conferences and evidence-based studies have been made. Guidelines published in 2008 recommend early treatment with broad-spectrum antibiotics (if the microbe is not known) and initial resuscitation (first

six hours) as the first line of treatment (113). Better diagnostics of the causing microorganism, in order to optimize the treatment, is desirable as the nature of the pathogens influence the host response (114). In addition to the treatment of the causing microbe, hemodynamic support is the most essential in the sepsis treatment. To maintain central blood flow and pressure, fluid therapy, vasopressors and inotrops are recommended. Additionally, supportive measures like mechanical ventilation, blood product administration, sedation and analgesia, glucose control, and corticosteroid administration may be used if needed (113).

Specific treatment of the complex and potential lethal inflammatory response in sepsis has been long sought. Numerous single mediators of inflammation have been targeted in the quest for a potent "wonder-drug". Promising results have been obtained from experimental studies, but results from clinical trials for instance targeting and neutralizing TNF-α have been disappointing (115). This paradox made one author write a review paper heading that spoke for itself: "Such stuff that dreams are made of: Mediator-directed therapy in sepsis" (116). Recombinant human activated protein C (rhAPC) has maybe been the most promising candidate for mediator directed therapy in sepsis, but clinical trials have not been able to provide sufficient evidence to justify general use of rhAPC in sepsis (117-119). Present guidelines recommend use of rhAPC in adult patients with high risk of death (113).

1.3 Inflammation in sepsis

The inflammatory response in sepsis is vast and complex. All the main cascade systems in the blood, the coagulation system and the fibrinolytic system (120, 121), the complement system (55, 122), and the contact system are activated (123), along with the main innate immune cells monocytes/macrophages and neutrophils (124, 125). Subsequently, the adaptive immune system will also be engaged in the development of the syndrome. In particular, the adaptive immune system has shown to shift from a T_H1- to a T_H2-cell driven response leading to profound immunosuppresion in the later phase (126), as well as to dampen the innate immune response in the early phase of sepsis (127, 128). Other cell and organ systems like endothelium (129), the endocrine system and the autonome nervous systems have shown to be important players in the

devastating sepsis game (130, 131). The development of an imbalance in the network of all these systems is thought to be responsible for the harmful loss of control of inflammation in sepsis. Crosstalk between the systems seems to be an integrated and pivotal part in the inflammatory drive. Examples of such crosstalks in inflammation are increasing and the research in this field is expanding. For instance has thrombin of the coagulation cascade been shown to directly cleave C5 of the complement system to C5a in the absence of C3 (132), indicating that C5a and TCC may be generated in the absence of upstream complement activation. C5a has been shown to induce the spark plug of coagulation, TF, both on endothelial cells and neutrophils (89, 133). The nervous system may both enhance and inhibit inflammation in a close crosstalk with innate immune cells (134). These are just a few of many examples of crosstalks important to be aware of when studying inflammation in sepsis.

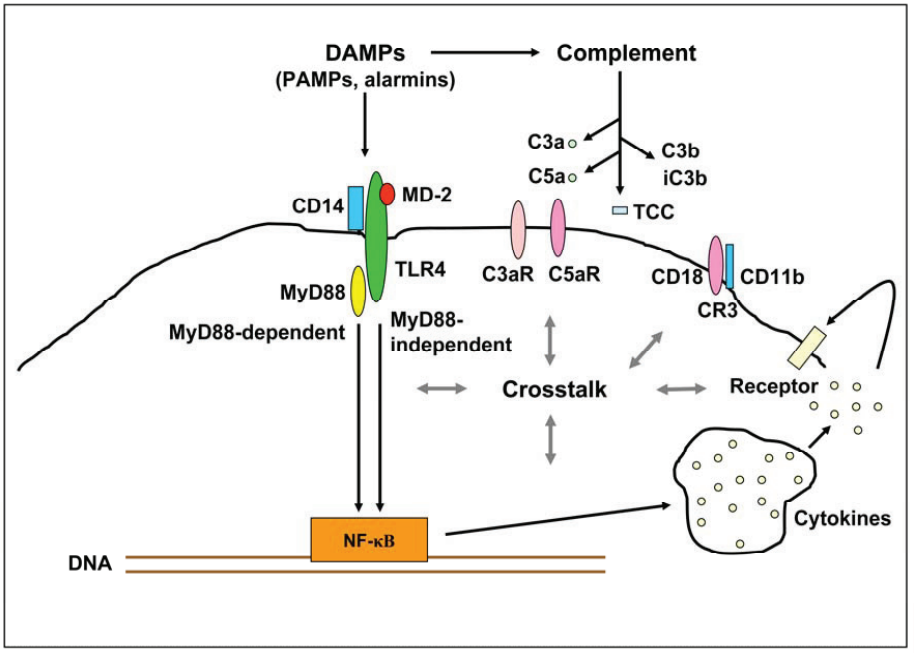

Figure 2. **Crosstalk in Gram-negative induced inflammation**

2 Aim

To investigate the effect of inhibition of Gram-negative induced inflammatory response by targeting two main innate immune upstream effector pathways, complement and CD14, in a pig model.

2.1 In vitro

1. To investigate the effect of known inhibitors of human complement in a porcine *in vitro* serum model, and compare the candidate inhibitors effect on different activators of complement using TCC as a marker of complement activation.

2. To establish a newly developed human *in vitro* whole blood model in porcine blood, and to use this model to investigate an anti-porcine CD14 antibody and *Orthopox*-viral derived complement inhibitors on *E. coli* LPS- or whole live bacteria-induced inflammatory response, and thereby the different roles of the CD14/TLR4/MD-2-complex and complement in this inflammatory response.

3. To study the potential of inhibiting *E. coli* LPS-induced inflammatory response by a *Cyanobacterial* LPS analog (CyP) known to bind to the (CD14)/TLR4/MD-2 LPS-receptor complex.

2.2 In vivo

1. To establish a porcine *in vivo* model of whole live *E. coli* bacteria-induced sepsis/inflammation with focus on mediators of the early inflammatory response induced by the bacteria.

2. To use the porcine *in vivo* model to investigate the inhibitory effect of the anti-porcine CD14 antibody on whole live *E. coli* bacteria-induced inflammatory response *in vivo*, including markers of hemostasis and granulocyte activation.

3 Material and methods

3.1 Introduction

The purpose of this part of the Thesis is to to give a cross-sectional and superior view of the material and methods used. For detailed description of reagents and methods please see the material and methods sections in each paper.

3.2 Inhibitors

Several unique inhibitors not commercially available were used in the studies. The *Orthopox*-viral encoded complement inhibitors VCP and SPICE were used in study **I** and **II**. The complement inhibiting monoclonal antibodies anti-factors B and D were used in study **I**. CyP, a product from blue - green algae was used in study **III.** The protease complement inhibitors C1-inhibitor and FUT-175 (study **I**), and the monoclonal anti-CD14 antibody (study **II** and **V**) were commercially available and purchased for the studies. The reagents not commercially available were provided by international collaborators.

3.3 Analyses

3.3.1 ELISA

The TCC assay is a so-called “sandwich” ELISA based on the human antibody aE11 as capture antibody in the wells. aE11 recognize a neoepitope on complement component C9 exposed after incorporation in the TCC (135), and thereby complement activation. aE11 and the detection anti-C6 antibody are shown to cross-react with porcine C9 and C6 respectively (136, 137), and the assay could therefore be used to detect porcine TCC (study **I-IV**). Normal human serum activated with zymosan and defined to contain 1000 arbitrary units (AU)/mL was used as standard, while zymosan activated porcine serum was used as positive control.

The porcine cytokines TNF-α, IL-1β and IL-8 (study **II-V**) were detected under the conditions used both *in vitro* and *in vivo*. IL-6, however, was detected *in vivo* (study **IV-V**) but only *in vitro* using a high dose LPS (study **III**). IL-10 (study **IV-V**), IL-12 and VEGF (study **IV**) were only detected *in vivo*. HMGB-1 was not detected in the studies. The cytokines were analysed by commercially available ELISA kits. The numbers of such kits available for pigs are limited compared to humans and mice. This restricted the number of cytokines possible to detect in the studies.

Citrate plasma was used in the analysis of coagulation and fibrinolysis. TAT was detected in a commercial available human ELISA proved to be applicable in porcine plasma (138) (study **IV-V**). PAI-1 was detected by a commercial available porcine PAI-1 ELISA (study **V**).

3.3.2 Flow cytometry

CD14 and wCD11R3, a porcine analog to human CD11b, was detected on granulocytes by flow cytometry both *in vitro* (study **II**) and *in vivo* (study **V**). It would have been interesting to look at these cellular markers on monocytes as well, in particular wCD11R3 as previous human studies have shown differences in upregulation and inhibition experiments on CD11b expression between granulocytes and monocytes (139). Unfortunately, it is not possible to separate pig monocytes and lymphocytes by gating in flow cytometry, in contrast to what is the case with human monocytes and lymphocytes.

Hematological parameters were analysed on one of the hospital's routine hematological instruments, which is in principle a flow cytometer.

3.3.3 Microbiological analyses

CFUs were measured both *in vitro* (study **II**) and *in vivo* (study **IV**). CFUs were readily detectable *in vitro* and the time-dependent effects of the inhibitors of complement and CD14 could be studied. The method seemed realiable for this purpose *in vitro*, as two

separate experiments from two different pigs were virtual identical (study **II**). *In vivo*, however, only trace amounts of bacteria were detectable by this method. Even after a large bolus of bacteria was injected, only a few bacteria were detected in the blood after 10 minutes (study **IV**). The method was therefore omitted in the *in vivo* study **V**.

A novel *E. coli* DNA detection analysis applied in study **V** was developed. Based on SeqA primers, *E. coli* DNA could be detected and quantified by real-time PCR. The primers were not *E. coli* specific as they also detect *Shigella* species and *Salmonella enterica* DNA, but this was not a problem for our purpose. CFU counts are very useful to detect living bacteria, while DNA analyses detect both living and killed/disrupted ones. The methods complement each other and it is important to be aware of their advantages and limitations.

LPS was detected by an endpoint chromogenic method in a *Limulus* Amebocyte Lysate (LAL)-assay (study **V**). The LAL-assay is based on a protein cascade which is activated by LPS and ultimately cleaves a peptide substrate which liberates a yellow substance (p-nitroaniline). The yellow colour can be measured directely. But as we measured LPS in serum which absorbs at the same wavelength as p-nitroaniline (405-410 nm), a diazo-coupling modification was used to change the detection colour to magenta (absorbs at 540-550 nm).

Alexa FLUOR 488 stained *E. coli* was injected into two pigs in study **V** and immunofluorescent histology of cryo-sections cut from the lung and liver biopsies was performed, and *E. coli* were qualitatively detected in the tissues.

3.3.4 Gelatine zymography

Matrix metalloproteinase (MMP)-9, a protease released by activated granulocytes and monocytes, was detected by gelatine zymography (study **IV** and **V**). Pictures were taken of the gels and activity of MMP-9 in the gels was quantified by special software. It was important to analyse samples that were going to be statistically compared in the same run, as inter-assay runs could differ substantially.

3.4 Models

3.4.1 *In vitro* model

The first part of the *in vitro* studies was conducted in serum. Serum was prepared from three healthy pigs and tested in Wielisa for assessment of complement functional activity in classical, lectin, and alternative pathway (140). The assay is based on the human monoclonal detection antibody aE11, known to cross-react with pigs (136, 137), and a lower serum dilution was used to optimize conditions when testing pig serum (137). The sera were pre-incubated in polystyren tubes at 37 ºC for five minutes with complement inhibitors and then activators of complement were added and incubated for another 30 minutes (study **I**). Complement activation was stopped after incubation by adding ethylenediaminetetraacetic acid (EDTA) and the sera were stored at -70 ºC before analyses.

The second part of the *in vitro* studies was conducted in whole blood. Whole blood from healthy pigs was anticoagulated with the specific thrombin inhibitor lepuridin (Study **II-III**). The method is described previously in human whole blood (64) and were proved to work in pig whole blood as well. The whole blood was incubated in polypropylene tubes with inhibitors of complement or the CD14/TLR4/MD-2 LPS-receptor for five minutes at 37 ºC and then *E. coli* LPS or whole *E. coli* bacteria were added and incubated for 10 (flow cytometry detection of CD14 and wCD11R3), 30 (TCC), 120 (TNF-α, IL-1β and TCC) or 240 (IL-8 and CFU experiments) minutes at 37 ºC. Complement activation was stopped after incubation by adding EDTA, plasma was prepared and the samples were stored at -70 ºC before analyses.

3.4.2 *In vivo* model

A pig model was developed to study Gram-negative bacteria induced inflammation *in vivo* (study **IV**). Norwegian farm pigs of out-bred stock (*Sus scrofa domesticus*) were used in the studies. In study **IV**, 30 kg pigs were used, and the model was slightly modified in study **V** where 15 kg pigs were used. They were aneasthetized and surgery conducted as described in study **IV**. The pigs were hemodynamically and respiratory

extensively monitored, including blood pressures (CVP, MAP, MPAP, and PAOP), respirator settings and blood gases. An important difference between the pigs and the humans, however, are found in the reticuloendothelial system (RES). The pig has resident macrophages in the lung (141), closely resembling the Kupffers cells found in the human liver. When administering *E. coli* IV to the pigs, we experienced a rapid rise in MPAP, not so commonly seen in human sepsis. The high MPAP experienced was probably due to mediators like prostacyclins and –glandins and leukotriens released by the lung macrophages (141), leading to vasoconstriction locally in the lungs. The increased pulmonary pressure was problematic for the model, as circulation was compromised. However, rescusitation with physiological saline water (volume therapy) (study **IV-V**) and occationally norepinephrine administration (study **V**) made the experiments possible to conduct. This difference between pigs and humans is important to be aware of when interpreting data from pig sepsis models, especially data on circulation physiology.

3.5 Statistical considerations

In the experiments where the data "spoke for themselves", no statistics were used. This applied to all the data in study **I** and the majority of the data in study **II** and **III.**

The non-parametric Mann-Whitney *U* test was used for a couple of data sets in study **III** and **V,** and is the non-parametric counterpart to the parametric two-sample *t* - test. In study **III**, the test was used on the data presented in Table 1. As there was skewness in these data, a normal distribution could not be assumed and thereby could parametric tests not be used (142). In study **V**, the Mann-Whitney *U* test was used on the data presented in Figure 7. Some of the data were below the "cut off" for the analysis; these data were given arbitrary values and could be used in a rank test. Parametric tests could not be applied to such data.

A two-sample *t* – test for independent samples were applied in study **II** (Figure 6) and **V** (Figure 2, 4 and 5). There were no skewness in the data and they could thereby be assumed to be normal distributed, although one might criticize this assumption as the number of experiments was low (n ~ 6) in all these data sets.

In study **IV** and **V** more complicated methods were applied. Several parameters were measured repeatedly over the time course of the *in vivo* experiments. Hence, the data were not independent of each other and tests which assume independence of the data, like the *t* – test, could not be applied. A mixed model was applied in study **IV**. It is a flexible modern form of regression analysis and is well suited to look at group effects developing over time. Both random (e. g. different baseline values) and fixed effects (e. g. group) are taken care of in such models (143). However, mixed models are complicated and we needed help from a statistician to conduct the calculations. In study **V** we used a two-way ANOVA with repeated measures. A mixed model as used in study **IV** could not be applied as the number of animals was too low. The main effect (treatment with anti-CD14) over time was tested (interaction treatment and time) between the treatment and the positive control group.

In all the tests in this Thesis, a significance level of $P < 0.05$ was chosen. In other words it was a 5 % chance of rejecting the null hypothesis when it was true, a so called Type I error (144). This is in general an accepted Type I error risk in medical statistics, but lower significance levels like $P < 0.01$ are sometimes seen, reducing the risk of such an error. However, the lower the significance level the greater the risk of making the other mistake in significance testing, the Type II error. The Type II error is to fail to reject the null hypothesis when it is in fact false. The propability of not making a Type II error is called power. The higher the power is the less the risk of making a Type II error. This can be achieved by a high sample size and/or precise measurements. In this Thesis the number of samples in both the *in vitro* and the *in vivo* experiments was limited due to limited amounts of the unique reagents (e. g. complement inhibitors and anti-CD14). The risk of making Type II errors was therefore, considering, probably greater than the risk of making Type I errors.

4 Summary of results

Paper I: The serine protease inhibitor FUT-175 inhibited porcine complement activation measured as TCC to baseline levels at all concentrations used for all the three activators of complement, HAIGG, zymosan and *E. coli*.The other serine protease inhibitor tested, C1-INH, inhibited complement completely at the highest dose added, and inhibited HAIGG- and *E. coli*-induced complement activation more efficiently at lower doses than was the case for zymosan-induced activation. The monoclonal anti-factor B dose-dependently and almost completely inhibited zymosan- and HAIGG-induced complement activation, while *E. coli*-induced complement activation was not inhibited by the antibody. The monoclonal anti-factor D inhibited complement activation by all three activators by approximately 50 %. The recombinant *Vaccinia*-virus complement control protein, VCP, was the most specific candidate inhibitor of complement tested. It dose-dependently and completely inhibited complement activation by all three activators. On a molar basis, taking into account the lowest dose of each inhibitor which gave the best inhibition, VCP was the most efficient complement inhibitor of the ones tested.

Paper II: An anti-porcine CD14 monoclonal antibody dose-dependently saturated CD14 on granulocytes in porcine whole blood. Anti-CD14 was then shown to dose-dependently and completely inhibit *E. coli* LPS-induced production of the proinflammatory cytokines TNF-α, IL-1β and IL-8. Complement was activated by whole live *E. coli* bacteria in porcine whole blood, in the same manner as shown for complement activation in porcine serum in study **I**. VCP was shown to dose-dependently and completely inhibit *E. coli* induced complement activation in whole blood and thereby confirming the potent complement inhibitory effect of the protein, shown in study **I**. SPICE, another *Orthopox*-viral derived complement inhibitory protein, was shown to be approximately five times more potent than VCP. wCD11R3, the porcine analog to human CD11b which together with CD18 forms the iC3b-binding CR3-phagocytosis reseptor, was shown to be dose-dependently upregulated by *E. coli* on granulocytes in porcine whole blood. The upregulation was dose-dependently and completely inhibited by VCP but not anti-CD14, showing that this upregulation was complement-dependent. Bacterial clearance measured as CFU was shown to be partly

complement-dependent as SPICE but not anti-CD14 inhibited the clearance. Whole *E. coli*-induced TNF-α and IL-1β was dose-dependently and significantly ($P < 0.05$ and $P < 0.01$ respectively) inhibited by anti-CD14 but not SPICE, IL-8 was dose-dependently and significantly ($P < 0.05$) inhibited by SPICE but not anti-CD14, showing that TNF-α and IL-1β were dependent on CD14 and IL-8 on complement. Thus, both complement and CD14 inhibition were shown to attenuate *E. coli*-induced inflammation in whole blood *in vitro*.

Paper III: CyP, a *Cyanobacterial* derived LPS analog, did not induce production of the proinflammatory cytokines TNF-α, IL-1β, IL-6 or IL-8 when incubated in porcine whole blood. *E. coli* LPS added in the same amount induced production of the same cytokines. CyP dose-dependently and completely inhibited *E. coli* LPS-induced production of TNF-α, IL-1β and IL-8 in porcine whole blood. CyP reduced whole live *E. coli* bacteria-induced cytokine production, but to a lesser extent than LPS-induced production. The best inhibitory effect was seen on IL-1β production, where CyP reduced the production by 44 %. CyP was found to be a moderate inductor of porcine complement measured as TCC, compared to LPS from *K. pneumoniae*, *S. typhosa* and *E. coli*. LPS from *N. meningitides* was found to be the least potent complement activator of the LPS types, including CyP, tested. At doses below 10 µg CyP/mL whole blood, the dose needed for complete LPS-induced cytokine inhibition, no complement activation was observed. In summary, the TLR4/MD-2 inhibitor, CyP, was shown to be an efficient inhibitor of *E. coli* LPS-induced inflammation in whole blood.

Paper IV: A pig model of acute sepsis with focus on the early inflammatory response and biomarkers of this response was developed in order to use it for interventional studies based on *in vitro* findings from study **I-III** in the future. Healthy 30 kg pigs were challenged with whole live *E. coli* bacteria either intravenously (IV) (n = 12) or intrapulmonary (IP) (n = 6). Control pigs received bacterial culture medium (n = 6 + 3). Haemodynamic compromise, as seen in sepsis and septic shock in humans, was also seen in this pig model. Aggressive fluid rescusitation was done in order to maintain MAP above 65 mmHg. Due to this, the pigs did not develop signs of hypoperfusion, like increased blood lactate. In the IV arm TNF-α ($P = 0.006$), IL-6 ($P = 0.005$), IL-8 (P

= 0.033), IL-10 ($P < 0.001$) and IL-12 ($P = 0.012$) increased significantly in the *E. coli* group compared to the culture medium group. IL-1β also increased, but failed to reach significance ($P = 0.063$). No significant differences between the cytokines were detected in the IP arm. A marker of coagulation, TAT, incresead significantly in the IV arm *E. coli* group compared to the control group ($P < 0.001$). MMP-9, an enzyme which is released from granulocytes and enzyme-activity increases during inflammation, increased in the *E. coli* group in both the IV and IP arm compared to the control group. The increase came earlier in the IP arm than in the IV arm. Complement activation and CFUs were only to a minor extent detected in this model. In conclusion, this pig model provided new insights into the early inflammatory response in experimental sepsis.

Paper V: The study was based on the pig *E. coli* sepsis model developed in study **IV.** Pigs were challenged IV with either whole live *E. coli* bacteria or *E. coli* LPS. The anti-CD14 antibody studied *in vitro* in study **II** was shown to completely and significantly saturate CD14 on granulocytes *in vivo* compared to the positive control group ($P < 0.0001$) and the saturation was sustained throughout the observation periode in both the *E. coli*-and the LPS-arm. TNF-α ($P = 0.032$), IL-1β ($P = 0.008$) and IL-6 ($P < 0.0001$) were significantly reduced by anti-CD14 in the *E. coli*-arm, while IL-8 was completely inhibited by anti-CD14. TNF-α, IL-6 and IL-8 were reduced in the same manner in the LPS-arm, while IL-1β values were around the lower detection limit in both the anti-CD14 and control groups. IL-10 showed a different time course in the anti-CD14 group compared to the positive control group in the *E. coli*-arm but not in the LPS-arm. In the *E. coli*-arm IL-10 increased later than the other cytokines, but reached levels higher than in the positive control group. MMP-9 increased in both the *E. coli*- and the LPS-arms, but not in the negative control groups. The increase came earlier in the LPS-arm. MMP-9 was significantly reduced ($P = 0.003$) after 120 minutes in the anti-CD14 group in the *E. coli*-arm, but not in the LPS-arm. wCD11R3 on granulocytes were significantly ($P = 0.008$) reduced after 180 minutes in the anti-CD-14 group in the *E. coli*-arm and the same trend was seen in the LPS-arm. There were no differences in LPS-levels between the anti-CD14- and positive control-groups in either arm of the study ($P = 0.81$ in the *E. coli*-arm). No difference was detected between the anti-CD14- and positive control groups ($P = 0.37$) in *E. coli*-DNA levels in the *E. coli*-arm. *E. coli*-DNA was not detected in the LPS-arm. The marker of coagulation, TAT, was significantly ($P = 0.011$)

inhibited by anti-CD14 in the *E. coli*-arm. The same was the case for the suppressor of fibrinolysis, PAI-1, which was significantly ($P = 0.005$) inhibited by anti-CD14 in the *E. coli*-arm. In summary, CD14-inhibition efficiently attenuated the proinflammatory cytokine response and granulocyte activation, reversed the pro-coagulant state, but did not interfere with LPS levels or bacterial counts in experimental *E. coli*-induced sepsis *in vivo*.

5 Discussion

5.1 Activators of inflammation

5.1.1 LPS

LPS of Gram-negative bacteria consists of a lipid A moiety, a short core oligosaccharide and an *O*-polysaccharide of variable length. LPS with *O*-polysaccharides is called smooth LPS, while LPS without *O*-polysaccharides is called rough LPS. The LPS molecule varies substantially among the Gram-negative bacteria and among different strains of each bacterium. Additionally, Gram-negative bacteria have several enzymes that can modify LPS in theire membranes (145). It is accordingly a vast diversity of LPS types.The purity of the LPS is another important feature to keep in mind when studying LPS responses through the TLR4 pathway. It is shown that contaminants, like lipoproteins, in crude LPS preparations signals through the TLR2- as well as the TLR4 pathway (146). The LPS used in study **II**, **III** and **V** was treated by phenol-extraction by the manufacturer and was so-called ultra-pure. It thereby only binds to and signals through the TLR4 receptor complex, which was the intention in this Thesis.

5.1.2 Whole live *Escherichia coli* bacteria

The use of LPS to mimic Gram-negative sepsis and septic shock is common and LPS is recognized as important in the pathogenesis of Gram-negative sepsis (147). However, live Gram-negative bacteria have properties that LPS can not mimic. We showed in study **II** that the bacteria devided when incubated at 37 ºC *in vitro* and additionally live *E. coli* bacteria were retrived after 240 minutes *in vitro* incubation in whole blood. Thus, dynamic features of the living bacteria in contact with the host can not be found in LPS models. Although LPS is pivotal, other ligands on Gram-negative bacteria could also contribute to the pathogenesis of inflammation and sepsis. It is for instance shown that LPS-deficient *N. meningitides* (LPS^-) can activate the complement system and induce complement mediated inflammatory effects (148). *N. meningitides* (LPS^-) have

also been shown to be able to induce large gene expression changes in human monocytes (149). In study **II**, we found that anti-CD14 inhibited production of LPS-induced IL-8 production in whole blood, but not whole live *E. coli*-induced IL-8. In study **III**, CyP was shown to efficiently inhibit LPS-induced proinflammatory cytokines to baseline levels, while the effect on *E. coli*-induced production of the same cytokines was moderate. We therefore argue that experiments with whole live bacteria, as conducted in this Thesis, give additional information to LPS in studies of Gram-negative inflammation and sepsis.

5.2 The inhibitors used

5.2.1 Complement inhibitors

A range of complement inhibitors have emerged over the last two decades, and the awareness of the potential of inhibiting complement in inflammatory diseases like sepsis is increasing (150, 151). Several promising studies have been conducted in rodent sepsis models. C1-INH has shown to protect against CLP-induced sepsis in mice (152), although much of the protective effect of this inhibitor is probably not complement mediated (153). Inhibition of Crry, a rodent C3 convertase inhibitor, induced lethal endotoxin shock in rats, showing the protective role of this membrane bound complement inhibitor (154). C5a is emerging as a key molecule in sepsis and inhibition of C5a in rodent sepsis models has shown a protective role, see for instance Huber-Lang et al. (155), or Flierl et al. (156). Anti-C5aR or anti-C5L2, both C5a receptors, have shown protective effects in a rat CLP sepsis model (157). In contrast to rodents, studies addressing porcine complement inhibition in general and in sepsis particular have been, with a few exeptions like Mohr et al. (158), scarce. In study **I,** this need was addressed as complement inhibitors of three main cathegories (serine protease inhibitors, monoclonal antibodies, and a recombinant protein) were tested as inhibitors of complement activation in a "pure" *in vitro* system in porcine serum. Interestingly, human reagents like C1-INH and murine reagents like anti-mouse factor B showed effect in pig serum. Particularly, VCP, a protein derived from the *Orthopox*-virus *Vaccinia*, showed impressing complement inhibitory effect on complement activated by three different potent complement activators (HAIGG, zymosan and *E. coli*). In study

II, porcine complement inhibition by VCP was confirmed in a more "complex" *in vitro* system, whole blood. Another *Orthopox*-viral derived protein tested in study **II**, SPICE, was shown to be an even more potent complement inhibitor than VCP. Thus, this Thesis has provided novel information on complement inhibitors which can be used in pig sepsis models and pig models of human diseases in general.

5.2.2 Inhibitors of the TLR4 complex

CD14 is a central molecule in the LPS-receptor complex. It was first thought that all LPS types was transferred by LBP to CD14 and that CD14 indiscriminatly delivered the ligand to TLR4, a process that concentrated the LPS signal (18). It is, however, revealed that CD14 is essential for proper recognition of smooth LPS, the commonest form of LPS expressed by Gram-negative bacteria, but not rough LPS (47). CD14 is thereby thought to be a highly specific receptor and responsible for at least part of the ligand specificity of the LPS-receptor complex (41). CD14 has thus been named "the smooth operator" for LPS reponses (48). LPS from *E. coli* strains are smooth (159). CD14 would according to these findings by Jiang and Gangloff (41, 47), be essential for recognition of and signaling by the *E. coli* LPS used in study **II**, **III** and **V** in this Thesis. Inhibiting CD14 in order to reduce smooth LPS-induced inflammatory responses might therefore be more effective than inhibiting TLR4. CD14 is also known to be a co-receptor for other TLRs in addition to TLR4, for instance for TLR2, a receptor for lipoproteins/peptides from all kinds of bacteria, and the intracellular TLR3 (43, 160). Although a recently published study showed impressive effect of an anti-TLR4 antibody in a murine model of *E. coli*-sepsis (161), one might claim that CD14 is a more upstream and general target for inhibition of inflammation than TLR4.

The anti-porcine CD14 antibody clone MIL-2 used in study **II**, **III** and **V**, has previously been found to inhibit binding of LPS (162). In this Thesis, the anti-CD14 monoclonal antibody was functionally characterized. It was found to bind to CD14 on porcine granulocytes and to be an excellent inhibitor of *E. coli* LPS-induced inflammatory responses both *in vitro* and *in vivo*. In study **II** and **V**, the antibody showed impressive inhibition of whole live *E. coli* bacteria-induced inflammation both *in vitro* and *in vivo*. Surprisingly, in study **V** new features of the antibody, like inhibition of TAT and PAI-1, markers of coagulation and fibinolysis respectively, were

revealed. To our knowledge, it is the first study to show that an anti-porcine CD14 antibody inhibit LPS- and whole *E. coli* bacteria-induced responses *in vivo*. The finding would be useful for further studies in the pig addressing Gram-negative induced inflammation.

CyP, a newly discovered inhibitor of TLR4/MD-2 derived from blue-green algae, shown to protect mice against LPS-induced shock (163), was tested *in vitro* in porcine whole blood in study **III.** CyP was for the first time shown to effectively inhibit *E. coli* LPS-induced production of central proinflammatory cytokines in porcine whole blood. However, the effect on whole *E. coli* bacteria-induced inflammatory response was more moderate.

5.3 Relevance of the models- advantages and limitations

5.3.1 The pig as a model animal

The pig was chosen as a model animal in this Thesis. Non-human primates are probably the supreme model animals for studies of human diseases. Monkeys are comparable in nearly all physiological and immunological aspects to humans (164). It is not prohibited by Norwegian law to use monkeys as laboratory animals. However, ethical, animal fascility and economical considerations make studies on non-human primates non-desireable and *de facto* impossible to conduct in Norway. Rodents like mice and rats are the most frequently used model animals for almost all studies of human diseases, but we argue that the pig has advantages over rodents in several aspects of sepsis studies. First, the pig is a large animal. Instrumentation, equipment like catheters, survailance, and intensive care treatment like volume rescucitation and medication closely resembles standard human critical care. Second, repeated blood sampling is not restricted in the same way as in smaller animals and at euthanasia tissue sampling is easy to conduct. Third, the anatomy and physiology of the pig closely resembles that of humans (165). Fourth, the pig is LPS sensitive in contrast to mice but similar to humans (166, 167), which is important studying Gram-negative induced inflammation. Fifth, there are high sequence and chromosome structure homology with humans (168), indicating that the majority of in particular the orthologous genes are conserved between the two species.

5.3.2 Pig i*n vitro* serum and whole blood models

The *in vitro* serum model is a pure, but simple system. It was very useful for the investigation of the potential porcine complement inhibitors in study **I**, as these experiments would not be biased by other components potentially present in more complex experimental systems. However, serum would not be useful for the investigation of inflammation as such, as inflammation is an extremely complex process with extensive crosstalk between cascade systems and a variety of cells. In serum, most of these are absent, for instance the coagulation system and blood cells to mention some.

In study **II** and **III**, a newly developed human whole blood model was tested and found to be feasible in pig whole blood. The model allows crosstalk between all the inflammatory systems in whole blood, with the exception of thrombin which is inhibited by the anticoagulant, lepirudin (64). The advantage of lepirudin is that it does not interfere with other biological systems in particular not the complement system (64), in contrast to other anticoagulants like EDTA, citrate and heparin (169). The whole blood *in vitro* model allowed studies on cytokine production and inhibition (study **II** and **III**), complement activation and inhibition (study **II** and **III**), upregulation and inhibition of a granulocyte marker (study **II**), and studies on bacterial growth and inhibition of the growth (study **II**). Although several aspects of inflammation were studied in the *in vitro* model, it has substantial inherent limitations. Important cell systems like endothel, which is known to be an important part of the inflammatory network in sepsis (170), and the solid organs are of course missing. The blood was incubated in plastic tubes, and was thereby in constant contact with an artificial surface during incubation. The plastic surface of the tubes is shown to activate the human complement system and complement mediated effects like upregulation of CD11b on phagocytes (171). The same was observed in study **II**, as a substantial background activation of complement was seen after incubation of porcine whole blood. Despite the limitations important to be aware of, the *in vitro* models used in this Thesis revealed essential information on the relative role of CD14 and complement in *E. coli*-induced inflammation and allowed studies of the effect of unique and rare reagents in limited amounts on this inflammatory response.

5.3.3 Pig *in vivo* model

As discussed in chapter 5.3.1, the pig has several advantages as a model animal. The pigs used in this Thesis was of outbred stock and of either sex, thus reflecting the diversity of sepsis patients better than inbred strains of male sex often used in rodent models (172). However, there are several limitations to our pig model. The pigs used in study **IV** and **V** were young, approximately 11 weeks old in study **IV** and 7 weeks old in study **V**, resembling humans in their adolescence. The pigs were healty prior to the experiments. In the clinical setting, patients experiencing severe infections and sepsis are often old and have pre-existing conditions like diabetes influencing the outcome of the disease. Pigs have a different RES than humans, with resident lung macrophages releasing mediators leading to pulmonary hypertension (141), as discussed in chapter 3.3.2. This is not as commonly seen in human sepsis, and is important to be aware of in particular when interpreting physiological parameters in pig sepsis models.

A limitation of both the *in vitro* and the *in vivo* models was the availability of specific porcine kits and reagents to detect biomarkers. We used porcine cytokine kits commercially available, but the numbers of such kits are limited compared to rodent and human kits. We used some human kits that cross-react with porcine epitopes, for instance the TCC ELISA (136), TAT (138), and VEGF (173). We also used kits, like HMGB-1, that had specificity for several species including pig and human. Kits using multiplex technology, which are extremely useful for detection of large numbers of human and mouse cytokines in small sample volumes, does not exist with pig specificity. Unfortunately, a human multiplex kit tested in our laboratory showed very little cross-reactivity with porcine cytokines (174), and could therefore not be used in this Thesis.

5.4 Sepsis and the models

An important question in all research is: Do we really study what we want to study? The questions may seem banal, but it is not. Projected to this Thesis the question would be: Did we really study sepsis? Obviously, the *in vitro* studies **I-III** was not sepsis studies according to the definition of sepsis outlined in chapter 1.2.1, but nevertheless brought

insight into aspects of the inflammatory response encountered in Gram-negative sepsis. The clinical course of sepsis is usually prolonged, over days to weeks. The observation time in our *in vivo* animal model was four to five hours. The observation time was limited by logistical challenges of these very resource-demanding experiments. We have therefore emphasized that early events in sepsis or septic shock are investigated in study **IV** and **V**. In the new pig sepsis model described in study **IV**, we tried two ways of bacterial challenge, IV or IP. The pigs challenged IV developed features of a systemic inflammatory response as seen in sepsis and this challenge mode was therefore continued in study **V**. IV bacterial challenge of often large doses infused over relatively short time is frequently used in sepsis models, but is equally frequently critizised. It is difficult to mimic the clinical situation with the IV challenge, as clinical sepsis develope slowly by intermittent release of bacteria from an infectious focus, in variable doses, and over an extended period of time (175). The IP challenge in study **IV** did not develop into a systemic inflammation as seen in sepsis, probably because of a compartmentalization of the infection and the inflammatory response to the lungs. Tissue compartmentalization is a well recognized feature of infections and sepsis (176). It is for instance shown that proinflammatory cytokines are restricted to the lung with unilateral pneumonia and not found in the contralateral lung (177). A systemic inflammatory response may occur with the compartmentilazed infection as source, but the limited observation time of study **IV** probably prevented that. IP challenge was therefore not conducted in study **V**. CLP might be the model that best mimic clinical sepsis (178, 179), and is frequently used in small animals. Peritonitis induced sepsis models are also conducted in pigs (167), and although CLP is possible to conduct and has been described in pig models (180), it is rarely used. A CLP model was not possible to use for us, because of limitations of the animal facilities and the possibilities of prolonged surveillance of the animals.

Because of the complexity of sepsis, Marshall et al. have claimed that there is no single "ideal model" of sepsis or shock (181). We have developed a large animal model (study **IV**) and used it for an interventional study (study **V**), and despite the limitations, it has provided novel and important information in particular on the early phase of sepsis and septic shock.

5.5 Inflammation in sepsis and the models

Sepsis is an extremely complex and heterogeneous dynamic syndrome involving most protein cascades, cells and organs in the body as described in chapter 1.3. Thus, shortcomings of *in vitro* and even *in vivo* models in sepsis studies are almost mandatory. An imbalance in the "inflammatory network" is depicted to be the major cause of the septic syndrome, an imbalance involving both exaggerated inflammation and immunosuppression (182). The DAMPs from either invading pathogens or damaged host tissue or both, initiate the inflammatory response in sepsis (7, 183). In our models PAMPs like LPS from *E. coli* and probably also alarmins from damaged tissue in the *in vivo* studies contributed to the inflammatory response observed.

An imbalanced cytokine response, often refered to as a "cytokine storm", of both pro- and anti-inflammatory cytokines is produced in response to the initiating DAMP recognition (184). In the *in vitro* studies **II** and **III** the proinflammatory cytokines TNF-α, IL-1β, and IL-8 were retrived, while IL-6 was only detected when blood was incubated with a high dose of LPS (study **III**). IL-10, VEGF, IFN-γ, and IL-12 were not detected under the conditions used *in vitro*. In the *in vivo* studies **IV** and **V**, IL-6 and IL-10 (study **IV** and **V**) and IL-12 (study **IV**) and VEGF (study **IV** and **V**) were retrived in addition to TNF-α, IL-1β, and IL-8, again showing that different experimental settings yield different results. The cytokines are often described to have distinct time profiles in experimental sepsis, for instance TNF-α and IL-1β are known to rise early (185), and in turn help induce production of other cytokines and chemokines like IL-6 and IL-8 (16). We showed the same cytokine pattern in study **IV** and **V**. The anti-inflammatory cytokine IL-10 is known be produced later in the inflammatory prosess, and high mobility group box-1 (HMGB-1) is another "late marker" in sepsis (186). We were not able to detect HMGB-1 in our *in vivo* model, probably because of the limited observation time. The anti-inflammatory cytokine IL-10, however, was detected in study **IV** and showed an interesting time course in study **V** discussed in chapter 5.6. As our models produced pro- and anti-inflammatory cytokines known to be central in the development of inflammation in sepsis (187), the models seem relevant for investigation of these aspects of inflammation.

As high levels of soluble TCC is shown to correlate to mortality in meningococcal sepsis in humans (188), we wanted to examine soluble TCC in our models. TCC was readily detected *in vitro* (study **I**-**III**), but with a distinct bacterial load-threshold of around 10^7 *E. coli* bacteria/ mL whole blood (study **II**). We were not able to detect TCC *in vivo* (study **IV**-**V**), except when a bolus of a high amount of bacteria was given to a pilot animal (study **IV**). Failure to reach a threshold of bacteria giving detectable soluble TCC *in vivo* in addition to lower sensitivity of the modified human TCC ELISA to porcine plasma was probably the reason for this. As wCD11R3, a phagocyte biomarker shown to be complement dependent *in vitro* (study **II**), was also detected *in vivo* (study **V**), and as it is known that complement activation occur locally on the PAMPs on bacteria and on alarmins on damaged tissue (8), we would therefore argue that complement activation also took place in our *in vivo* model and contributed to the inflammatory response.

Time dependent development of the dynamic sepsis syndrome, with temporal modulation of mediators of the inflammatory response, adds to the complexity (189). In this Thesis many of the central systems present in the inflammatory network was examined and the temporal dynamics of several mediators were shown within the limits of our observation times. Pro- and anti-inflammatory cytokines were shown to have distinct time courses (study **IV**-**V**) and these time courses differed some between the LPS- and *E. coli* challenge groups (study **V**). LPS is previously shown to fail to induce IL-1β production *in vivo* in humans (190), and the same was demonstrated in pigs in study **V**. Whole *E. coli* bacteria, on the other hand, induced substantial amounts of the cytokine in our *in vivo* model (study **IV** and **V**). The markers of coagulation (TAT) and fibinolysis (PAI-1) (study **IV** and **V**)**,** the neutrophil activation marker wCD11R3 (study **II** and **V**), and MMP-9, an enzyme released by activated neutrophils and monocytes, (study **IV** and **V**) also showed time-dependency with similar trends in the LPS- and *E. coli* groups.

Not only are many cell and cascade systems involved in the pathogenesis of sepsis, but crosstalk between these systems seems to be a new emerging field in research to try to understand the pathogenesis (191, 192). Recent studies have revealed crosstalk between the complement system and TLR4. In decay accelerating factor (DAF) $^{-/-}$ mice, DAF is a membrane-bound complement inhibitor, complement via C3a and C5a were shown to

modulate the TLR4-induced cytokine response *in vivo* (193). The authors claim the absence of DAF may mimic overwhelming complement activation as seen in Gram-negative sepsis, and therefore such a crosstalk may also have occurred in our models. Additionally, we showed that wCD11R3, a porcine analog of CD11b which together with CD18 forms the phagocytosis receptor CR3, was upregulated on neutrophils upon stimulation with *E. coli in vitro* and *in vivo* (study **II** and **V**) and LPS *in vivo* (study **V**). The upregulation was totally dependent on complement *in vitro* (study **II**) and partly dependent on CD14 *in vivo* (study **V**), demonstrating the close relationship and possible crosstalk between complement and CD14/TLR4. Importantly, this also indicate the rational for inhibition of these upstream effector systems The possible crosstalk in these findings were interesting in the light of the findings by van Bruggen et al., where CR3 was shown to be essential for uptake of the Gram-negative bacteria Salmonella enterica and LPS-signaling via TLR4 was essential for the subsequent killing of the bacteria via production of ROS by the NADPH oxidase in the phagosome (194).

Coagulopathy as well as decreased fibinolysis are well known features of sepsis (195, 196). Both of these hemostasis abnormalities, leading to the septic "procoagulant phenotype", were detected in our *in vivo* model as both a marker for coagulation (TAT) and a marker for inhibition of fibrinolysis (PAI-1) increased (study **IV** and **V**). We do not know if the complement-coagulation crosstalk outlined in chapter 1.2.5 occurred in our model, but it is possible. Additionally, crosstalk between LPS activated platelets through TLR4 and neutrophils are described (197). The binding of these platelets to neutrophils leads to neutrophil activation and degranulation, demonstrated among other things by MMP-9 release. DNA is also released and subsequent formation of DNA-composed extracellular traps to ensnare bacteria in septic blood occurs. In study **IV** and **V** we demonstrated MMP-9 release in our *in vivo* model and that the release was partially CD14 dependent (study **V**). This dependency might reflect such a CD14/TLR4-mediated platelet-induced neutrophil activation.

In summary, the models in this Thesis revealed several central biomarkers of inflammation and their relative dependency on complement and CD14, as well as their relative time-dependency *in vivo*. The studies also exhibited crosstalk in the inflammatory network as seen in human clinical sepsis. Put together, the findings

enlightened important aspects of inflammation and showed that our models were relevant for studies of inflammation in sepsis.

5.6 New treatments in sepsis and possible clinical application of the results

Single downstream mediator neutralization to dampen inflammation, like TNF-α or IL-1β inhibition, have failed as a treatment strategy in sepsis (198). The same is the case for neutralization of LPS of Gram-negative bacteria (199). Regarding LPS inhibition, the principal differences between LPS and whole Gram-negative bacteria may explain some of the failure to translate promising preclinical endotoxin studies to clinical trials (200). In general, factors like heterogenous patient populations in age, inclusion time and severity of sepsis, have obviously contributed the treatment-trial failures (100, 201). The many disappointments of anti-inflammatory therapies in sepsis have led some researchers to ask the question if mortality in sepsis is not derived from an uncontrolled proinflammatory response (202). However, the complex interplay between the many pathogenic mediators, probably impossible to dampen with single target approaches, might be the main reason for the failures (198, 203).

Despite the complexity of sepsis and the vast amount of biomarkers, including cytokines, complement- and coagulation activation products, enzymes, ROS etc. correlated with development, severity or mortality of the syndrome, the expression of all these mediators is regulated through a relative small number of signalling pathways (204). Targeting upstream PRRs seems to be a better approach than single mediators of the septic inflammation. These central effector-pathways, which recognizes DAMPs (PAMPs and alarmins), initiates inflammation (14, 65, 205). Inhibing the upstream inducers instead of the downstream mediators of inflammation in sepsis, is an alternative treatment regimen (28, 151, 204, 206). Inhibition of two of these central upstream inducers of inflammation, complement and CD14/TLR4, was examined in this Thesis. We found that the inhibition led to reduction of a range of proinflammatory cytokines *in vitro* (study **II** and **III**) and *in vivo* (study **V**). The anti-inflammatory cytokine IL-10 clearly differed from the proinflammatory cytokines *in vivo*. In the *E. coli*-arm in study **V** the increase was delayed, but reached levels higher than in the positive control group. This could be beneficial in sepsis as IL-10 dampens the

inflammatory response and may enhance the effect of antibiotics (207). Activation of hemostasis (study **V**) and granulocytes (study **II** and **V**) was also inhibited *in vitro* and *in vivo* by targeting these two upstream danger sensor systems. Not only changes induced by LPS, but also largely those induced by whole live *E. coli* bacteria were inhibited.

There is a concensus in the scientific community that systemic inflammation induced by the host's response to DAMPs, PAMPs on invading pathogens and alarmins on damaged tissue are harmful and contribute to the development of severe sepsis and septic shock (202). The findings in this Thesis suggest that inhibition of CD14/TLR4/MD-2 and complement should be regarded as an early treatment approach in sepsis.

5.7 Future perspectives

The hypothesis of upstream inhibition of complement to dampen parts of the Gram-negative bacteria-induced inflammation has been demonstrated *in vitro* (study **I** and **II**). Inhibition of another upstream danger sensor, the TLR4/CD14/MD-2 complex, by anti-CD14 (study **II**), or an inhibitor of MD-2/TLR4, CyP (study **III**), showed additional anti-inflammatory effects to complement inhibition *in vitro*. The anti-inflammatory effect of anti-CD14 was confirmed and extended *in vivo* (study **V**).
The potent inhibitory response of CyP on LPS- but not E. coli-induced inflammatory response would be interesting to investigate further, and a study of the effects of two different fractions of CyP on this response is on its way. A continuation of the anti-CD14 study in vivo would be to look at inflammatory biomarkers including gene-regulation in the tissues and compare them with the results in blood obtained in study V. This could point to the relative role of the solid organs in relation to the blood in systemic inflammation, and reveal if anti-CD14 has any inhibitory effect on inflammation in the tissues. If so, tissue targeting of anti-CD14 could be a treatment approach. As complement inhibitors and anti-CD14 were shown to have complementary and differential inhibitory effects *in vitro* in pigs, and as a pronounced inhibitory synergism is previously shown *in vitro* in humans (64, 139, 171), it would be extremely interesting to investigate a combination of a complement inhibitor and anti-CD14 or

CyP, *in vivo* in our pig model. Unfortunately, an available large scale produced specific complement inhibitor working in pigs has been impossible to provide up till now.

6 Conclusions

1. The *Orthopox*-viral complement control proteins VCP and SPICE were shown to be excellent inhibitors of complement in porcine serum and whole blood *in vitro*. The proteins also inhibited upregulation of wCD11R3 (part of the phagocytosis-receptor CR3) and production of the chemokine IL-8 in whole blood, and thereby showed that these two biomarkers were complement dependent.

2. The complement inhibitor SPICE was shown to inhibit clearance of *E. coli* in porcine whole blood *in vitro*, thereby showing that the clearance is partly complement-dependent. The anti porcine-CD14 antibody did not influence clereance of *E. coli* either *in vitro* or *in vivo* in the pig, showing that the LPS-receptor is not responsible for bacterial clearance.

3. Inhibition of the CD14/TLR4/MD-2 LPS-receptor complex by either anti-CD14 or CyP was shown to be an efficient way to reduce production of the *E. coli* LPS-induced proinflammatory cytokines TNF-α, IL-1β and IL-8 *in vitro*. Anti-CD14 was also shown to inhibit *E. coli* LPS-induced TNF-α, IL-6 and IL-8 production *in vivo* in pigs.

4. Inhibition of CD14 reduced whole live *E. coli* bacteria-induced production of TNF-α and IL-1β *in vitro* and *in vivo* in the pig, and IL-6 *in vivo*, while production of IL-8 was inhibited *in vivo* but not *in vitro*.

5. Inhibition of CD14 reduced *E. coli*-induced upregulation of wCD11R3 *in vivo*, but not *in vitro*. Both TAT and PAI-1 and thereby the procoagulant state seen in sepsis was inhibited *in vivo* by anti-CD14.

In summary, complement and CD14 were shown to be important upstream danger sensing molecules mediating the host's inflammatory response to the Gram-negative bacteria *E. coli in vitro* and anti-CD14 showed an impressive inhibition of a broad spectrum of inflammatory mediators *in vivo* in a pig model. Important differences in the inflammatory response between challenge with *E. coli* LPS and whole live *E. coli* bacteria and between the *in vitro* and the *in vivo* system, were revealed. This emphasizes the importance of carefull interpretation of the model used in respect to the potential effect in a human clinical setting. Inhibition of complement and CD14 are promising therapeutic targets to dampen the overwhelming inflammatory response seen in sepsis, and may be useful in a clinical setting in the future.

7 Reference list

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8 Papers I-V

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